

AWARD NUMBER: W81XWH-15-1-0096

TITLE: Dietary Approaches to Protect Against Eye Blast-Induced Oxidative Stress and Vision Loss

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REPORT DATE: November 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

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1. REPORT DATE November 2016			2. REPORT TYPE Final		3. DATES COVERED 30 Sep 2015 - 29 Sep 2016	
4. TITLE AND SUBTITLE Dietary Approaches to Protect Against Eye Blast-Induced Oxidative Stress and Vision Loss			5a. CONTRACT NUMBER			
			5b. GRANT NUMBER W81XWH-15-1-0096			
			5c. PROGRAM ELEMENT NUMBER			
6. AUTHOR(S) Tonia S. Rex			5d. PROJECT NUMBER			
			5e. TASK NUMBER			
			5f. WORK UNIT NUMBER			
E-Mail:						
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Office of Sponsored Projects 1400 18 th Ave South Nashville, TN 37212					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S)	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT The premise of this study was that oxidative stress contributes significantly to the progressive cell death and neuronal degeneration that we detect after ocular trauma and, thus, treating with Vitamins C and E or a ketogenic diet would be protective. We have completed at least one cohort for each experimental condition and plan to repeat the studies to confirm our findings. We expect the project to be complete in another year, if we continue it. Preliminarily our results suggest that removal of Vit.C is detrimental to outcomes after blast exposure, however, additional Vit.C and Vit.E in animals that already produce sufficient levels, has no protective effect. The ketogenic diet also did not elicit a protective effect after blast. Rather, we detected an increase in the inflammatory cytokine, IL-1 and a decrease in the oscillatory potential of the electroretinogram independent of trauma. In summary, neither diet appears to be protective, however, we may have identified IL-1 as an important mediator of vision loss after ocular trauma.						
15. SUBJECT TERMS						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC	
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)	

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1. Introduction:

We previously detected an increase in nitrotyrosine immunolabeling in the inner retina prior to the onset of cell death, visual function deficits, or optic nerve degeneration in the C57Bl/6 mouse. This suggests that oxidative stress may play a role in these processes in the retina and optic nerve after ocular trauma as it does in the brain after traumatic brain injury. In order to test this hypothesis we tested the effect of depletion and supplementation of antioxidants and antioxidant enzymes. The ultimate goal of this study was to identify a dietary intervention that could protect Service Members from vision loss after neurotrauma by a simple modification of their MREs. The proposed experiments are multi-year investigations and thus what we report here are our preliminary data on this project with the hope of repeating these experiments in the future to confirm the findings.

2. Keywords:

oxidative stress, antioxidants, vitamin C, vitamin E, ketogenic, Gulo^{-/-}, SOD2, electroretinogram, visual evoked potential, sterile inflammation, interleukin-1

3. Accomplishments:

Task 1. Determine the efficacy of pre-treatment with antioxidants in preserving vision after eye blast trauma.

Prior to initiating therapy studies we assessed the effect of antioxidant deficiency in order to better understand the role (or lack thereof) for oxidative stress in vision loss that occurs after ocular trauma. The vast majority of people consume sufficient quantities of vitamin C (Vit. C) to avoid scurvy. However, most people do not consume optimal levels of Vit. C based on lack of saturation of the Vit. C transporters at the blood brain barrier. Data is accumulating that these less than optimal levels of Vit. C may increase susceptibility to neurological disease/damage, including Alzheimer's disease. Unlike people, mice contain an enzyme that allows them to produce Vit. C. We obtained mice that lack both copies of the gene encoding for this enzyme (Gulo^{-/-}), maintained them on low Vit. C and exposed them to the blast injury. In another experiment, we exposed mice lacking one copy of superoxide dismutase 2 (SOD2^{+/-}) to blast injury. We then assessed vision in both strains as compared to wild-type mice.

In order to demonstrate that the Gulo^{-/-} mice maintained on a low vitamin C diet were actually deficient in vitamin C, we measured vitamin C levels in the brain and liver (**Fig. 1A,B**). As expected, we detect significantly less vitamin C in the brain and liver of mice maintained on a low vitamin C diet. The mice were otherwise healthy. Surprisingly, despite the lower vitamin C levels, there was no increase in the oxidative stress marker, MDA in either tissue (**Fig. 1C,D**).

We previously reported that the oscillatory potentials (OP) of the electroretinogram (ERG) are decreased after blast injury. Surprisingly, we did not detect a decrease in the OP in

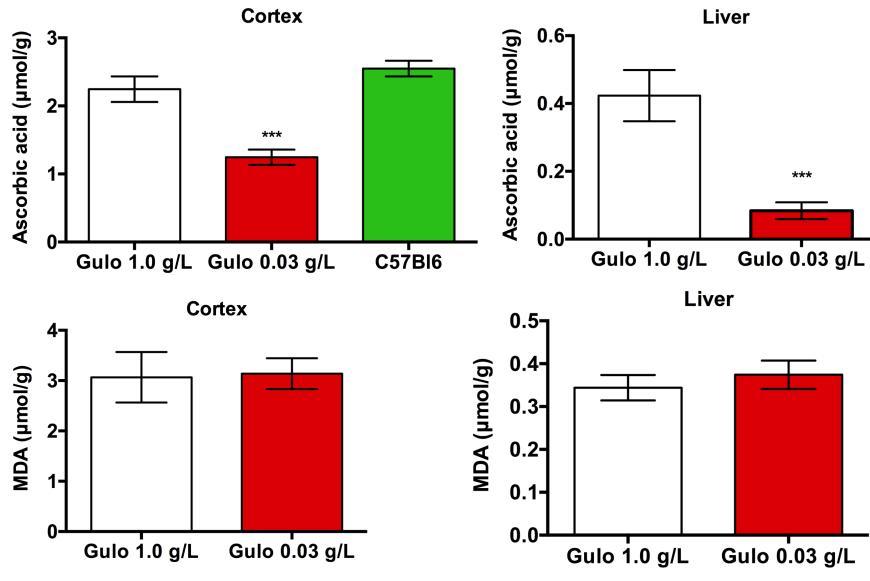


Figure 1. Vitamin C, not MDA levels altered by maintaining mice on low Vitamin C. A, B) Vitamin C levels in the A) cortex and B) liver of wild-type (C57Bl/6) and Gulo^{-/-} mice maintained on normal or low vitamin C. C, D) MDA levels in the A) cortex and B) liver of Gulo^{-/-} mice maintained on normal or low vitamin C.

either the low vitamin C Gulo^{-/-} or the SOD2^{+/-} mice after blast as compared to sham controls (**Fig. 2**). However, when we compared the results to wild-type mice we discovered that baseline OP values for both the Gulo^{-/-} and

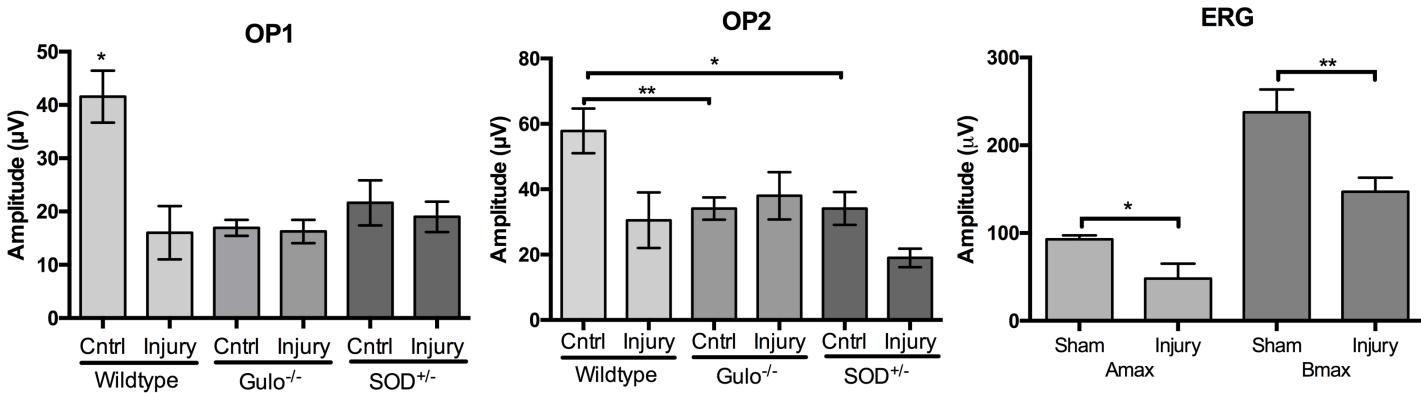


Figure 2. The ERG is decreased in mice lacking a full ability to respond to oxidative stress. A, B) The OP is decreased after blast in wild-type mice and is decreased in low vitamin C ($Gulo^{-/-}$) and $SOD^{+/-}$ mice regardless of injury. A) OP1, * control (no injury) OP1 amplitude was statistically different from all other groups. B) OP2, control OP2 amplitude was only statistically different from the control $Gulo^{-/-}$ and $SOD^{+/-}$ mice. C) Both the amax and bmax are decreased in $SOD^{+/-}$ mice despite being unaffected by blast in wild-type mice (ref), suggesting removal of an endogenous protective mechanism.

$SOD2^{+/-}$ mice were decreased to the levels detected in post-blast wild-type mice (Fig. 2). So, **deficiency in antioxidants causes a decrease in the OP in the absence of trauma and trauma does not cause a further deficit**. Further, we measured a decrease in the ERG a wave and b wave amplitudes in the $SOD^{+/-}$ mice (Fig. 2C), suggesting that $SOD2$ may serve an endogenous protective mechanism after blast. We are currently repeating these studies to confirm these results.

Next, we treated wild-type C57Bl/6 mice with high doses of Vit. C and Vit. E delivered in their drinking water beginning one month prior to blast and continuing until collection. We chose to treat with both antioxidants because both are powerful antioxidants and Vit.C recycles Vit. E, allowing it to continue to act. We are currently measuring the Vit. C levels in the cortex and liver and expect to have this data soon. We

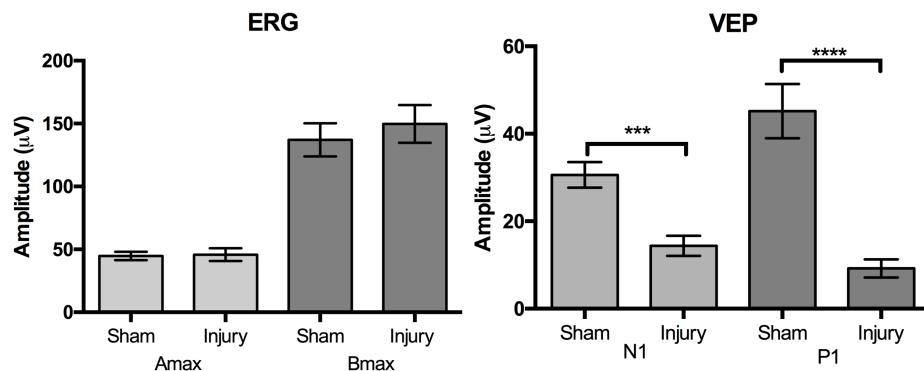


Figure 3. Mice given high levels of Vit. C and Vit. E show no change in the ERG amax or bmax, similar to normal controls (A). However, both show decreases in the pERG (B) and the VEP (C) suggesting that these antioxidants were unable to overcome damage to the RGCs and optic nerve.

performed electroretinograms (ERG) to assess photoreceptor and retinal interneuron function and detected no

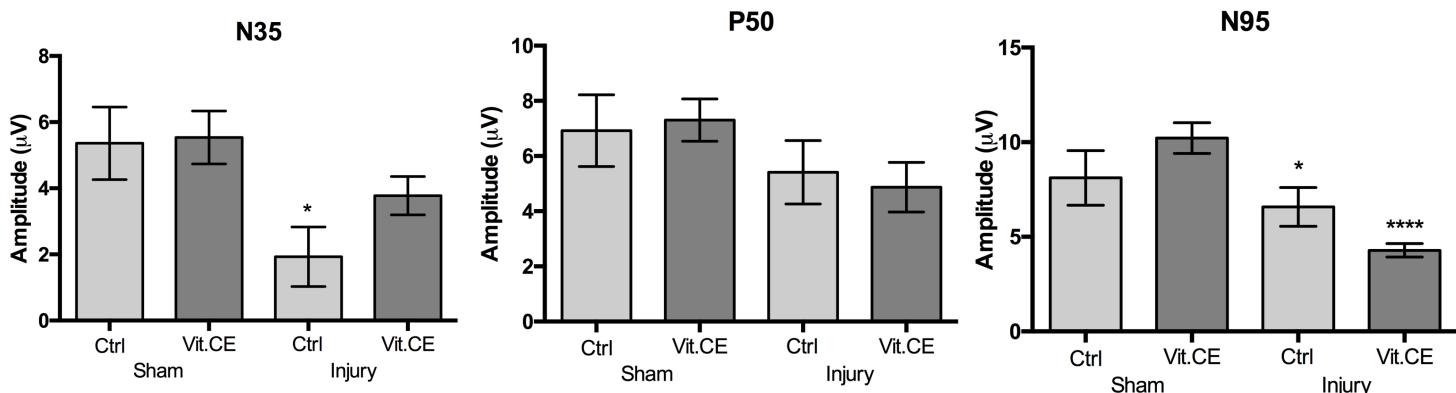


Figure 4. Amplitudes of the pERG waveforms in sham and post-injury mice maintained on a control diet or high Vit. C and Vit. E. A) N35 waveform, B) P50 waveform, and C) N95 waveform.

difference between sham and blast mice, consistent with our previous findings in wild-type mice on normal diets (**Fig. 3A**). Using funds from this grant we were able to purchase a new electrophysiology system that allows us to perform pattern ERGs (pERG) and visual evoked potentials during the same session as the ERG measurements. Interestingly, we detected a decrease in the amplitudes of both the N1 and P1 waveforms of the VEP after blast in the mice maintained on a high Vit.C and Vit. E diet (**Fig. 3B**). The VEP measures activity in the visual cortex and thus requires a completely functional circuit from photoreceptors onwards. Since the ERG looks normal, a deficit in the VEP points to dysfunctional signaling downstream of the retinal bipolar cells. The fact that we detect a deficit in the VEP in the mice maintained on a high Vit. C and Vit. E diet suggests that

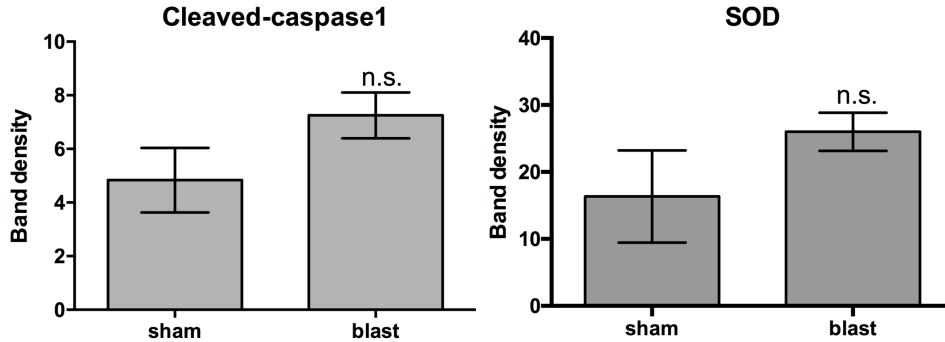


Figure 5. There is a trend for increased cleaved caspase-1 in injured retinas despite increased levels of Vit.C and Vit. E (A). We expect this to reach significance once more animals are added. There is a trend for an increase in SOD after injury, suggesting an endogenous protective mechanism, matching the preliminary data in **Fig. 2**.

even pre-treatment with these antioxidants is insufficient to protect the visual system against trauma-induced damage. To further narrow which cells may be responsible for the decrease in the VEP we performed pERGs. The pERG measures activity at the level of the retinal ganglion cell body. We detected a decrease in the amplitudes of the N35 and N95 waveforms of the pERG in wild-type mice maintained on a control diet (**Fig. 4 A,C**). There was no difference in the P50 waveform amplitude, but it is expected that as we increase the number of mice assessed, a statistically significant decrease might be detected (**Fig. 4B**). Interestingly, we did detect preservation of the N35 waveform amplitude in the mice that were treated with Vit.C and Vit. E. (**Fig. 4A**). In contrast, we detected a further decrease in the amplitude of the N95 waveform (**Fig. 4C**). The apparent preservation of the N35 could be due to the small number of control diet mice used in this study, which had, potentially, abnormally low values. We plan to repeat these studies to determine if these preliminary results will hold or if the effect will go away once larger numbers of animals are assessed.

Finally, to assess cell death and oxidative stress in the retinas of high Vit. C and Vit. E treated mice, we performed Western blot analysis for cleaved-caspase 1 and SOD2 (**Fig. 5**). We detect a trend for increased cleaved caspase-1 and SOD2 after blast. We expect that as we add more mice to this study that these trends may reach statistical significance. The caspase-1 data matches with data from our laboratory on mice maintained on a control diet (**Fig. 10**) and thus again, suggests a lack of a protective effect. The trend for an increase in SOD2 after injury suggests, as does the data in **Fig. 2**, that SOD2 may serve an endogenous protective role in the retina

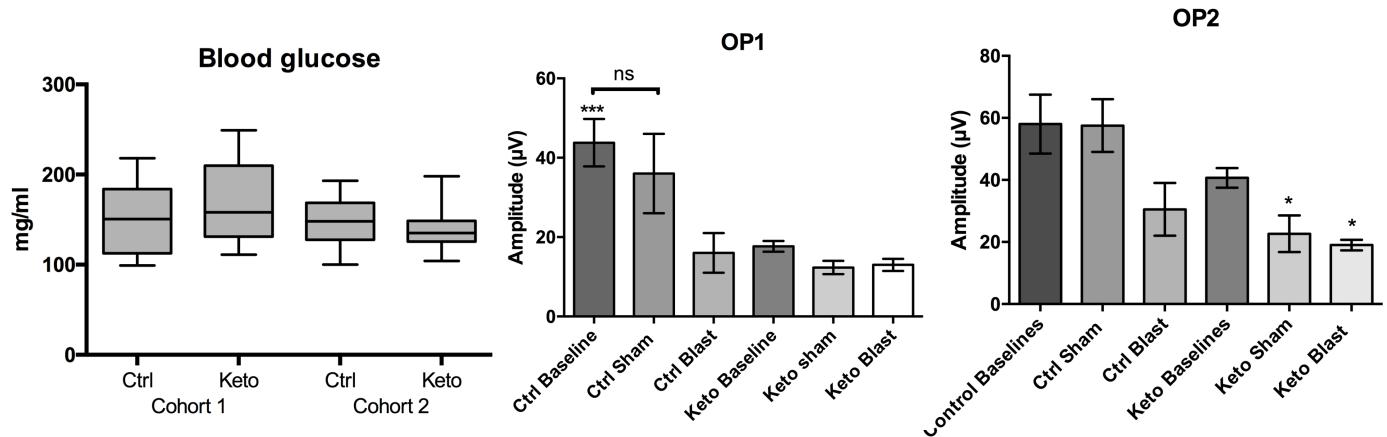


Figure 6. A) Mice on a ketogenic diet gained weight but did not develop diabetes. B, C) Mice on a control diet had decreased OP1 (A) and OP2 (B) amplitudes. Mice on a ketogenic diet had decreased OP amplitudes prior to injury that decreased further after blast.

after injury.

Task 2. Determine the efficacy of pre-treatment with a ketogenic diet in preserving vision after eye trauma.

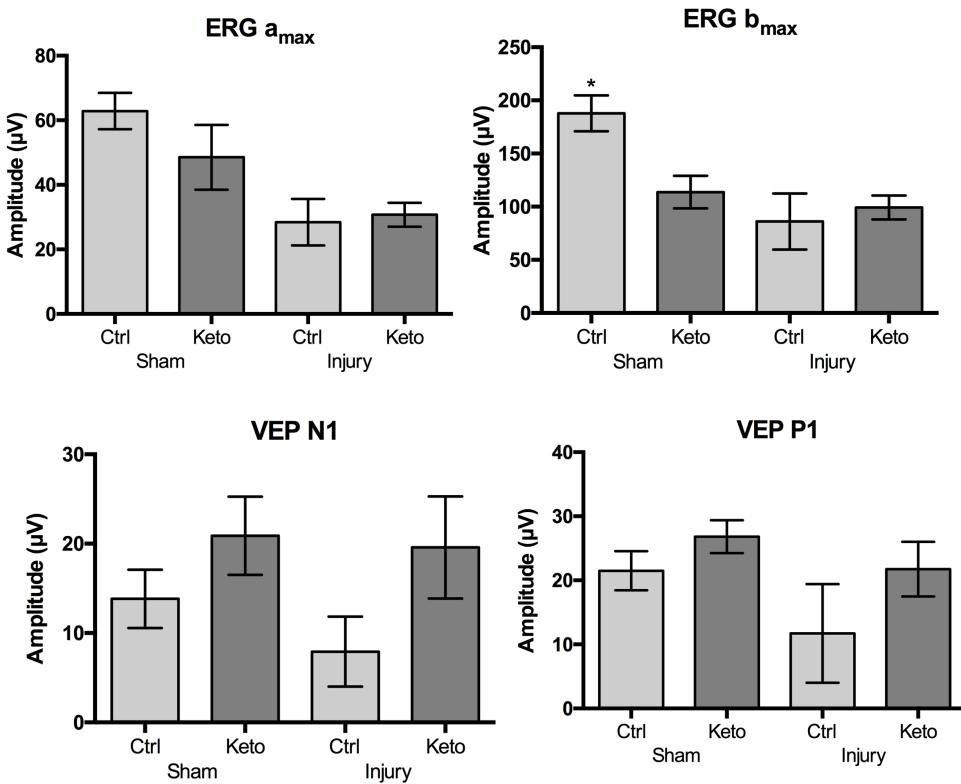


Figure 7. No statistically significant difference between wild-type and ketogenic mice in the amplitudes of the ERG a wave (A), b wave (B), VEP N1 (C), or VEP P1 (D). Error bars represent S.E.M., showing the low n and high variability of the assay.

to mice maintained on the control diet (**Fig. 6A**).

In cohort 2 mice, we measured ERG, pERG, and VEP to assess a protective effect. Surprisingly, in this cohort of mice we detected a decrease in the amplitudes of both the a-wave and b-wave of the ERG (**Fig. 7A,B**). The decrease in the a-wave amplitude was not significant (**Fig. 7A**) and we expect that this result will not hold once more mice are assessed (there were 5 or fewer mice in each group). There was also a trend for a decrease in the N1 and P1 waveforms of the VEP in the mice on the control diet after injury (**Fig. 7 C,D**). In contrast, the mice fed a ketogenic diet had N1 and P1 amplitudes after injury that were comparable to sham levels (**Fig. 7**

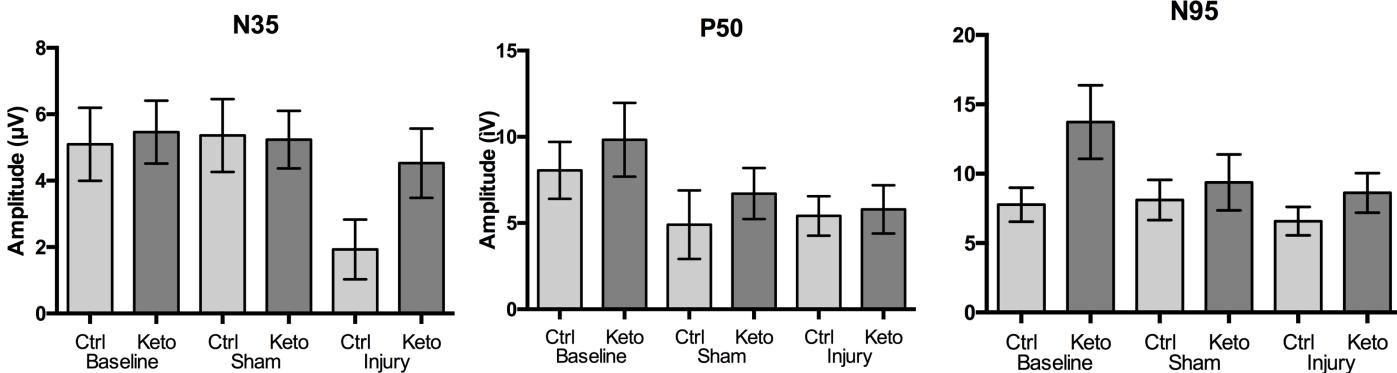


Figure 8. Amplitudes of the pERG N35 (A), P50 (B), and N95 (C) waveforms. A ketogenic diet appears to preserve the N35 aspect of the pERG, but otherwise no statistically significant difference was detected.

Mice were maintained on a ketogenic diet or matched control diet for four weeks prior to injury and for an additional month after injury (cohort 1). Some of the mice gained a large amount of weight, however, when pooled together there was no difference in blood sugar levels (**Fig. 6A**). Similar to the results with the low vitamin C diet in the *Gulo*^{-/-} mice, the mice maintained on a ketogenic diet had a lower OP1 amplitude regardless of presence of injury (**Fig. 6B**). A similar decrease was also detected in the OP2, however, in this case, there was a further reduction after blast (**Fig. 3C**). The number of animals with ERG recordings was very low due to issues with their weight, so we repeated this experiment and only pre-treated with the ketogenic diet for 2 weeks prior to blast exposure (cohort 2). These mice also had comparable blood glucose levels

C,D). We also detect preservation of the VEP N35 waveform amplitude after injury in mice maintained on a ketogenic diet (**Fig. 8A**). However, as mentioned above, this could be due to an underestimation of the amplitude in the control diet injured mice. We need to repeat this study. No differences between groups was detected for the P50 or N95 aspects of the VEP (**Fig. 8B,C**).

We performed a multiplex ELISA to determine if inflammation after injury was altered by a ketogenic diet.

The major change we detected was an increase in IL-1alpha in both sham and blast mice maintained on a ketogenic diet as compared to sham mice on a control diet (**Fig. 9**). The increase appears to be beyond that caused by blast in mice maintained on a control diet. We are repeating this experiment now. It further caused us to explore other related pro-inflammatory cytokines. We assessed levels and localization of multiple inflammasome pathway proteins in wild-type mice maintained on a control diet and exposed to blast (**Fig. 10**). We detected an increase in the number of cells and total levels of the caspase that mediates this pathway (caspase 1; **Fig. 10A-D**). Further, we detected an increase in the ratio of cleaved to total caspase 1, indicating activation of this enzyme after trauma (**Fig. 10D**). The two major by-products of activated caspase 1 are IL-1beta and IL-18. We detect an increase in both (**Fig. 10E, G, H**). Finally, similar to the results from the ketogenic study, we detect an increase in IL-1alpha (**Fig. 10I**). IL-1 alpha is active in both its pro- and cleaved forms and thus could be the initiator of the response. As shown in Figure 5J, oxidative stress can also feed into this pathway thus the antioxidant studies are still very relevant. Activation of this pathway can lead to cell death by over activation of the pannexin channel, leading to pyroptosis (**Fig. 10J**). We are exploring potential therapeutic interventions for this pathway including treatment with IL-1 receptor (IL-1R) antagonist since both

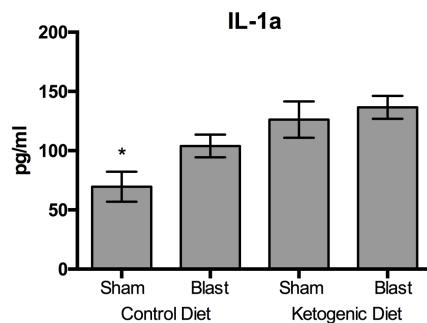


Figure 9. The pro-inflammatory cytokine, IL-1alpha is increased in the retina after ocular trauma and is increased with or without injury in the retinas of mice on a ketogenic diet.

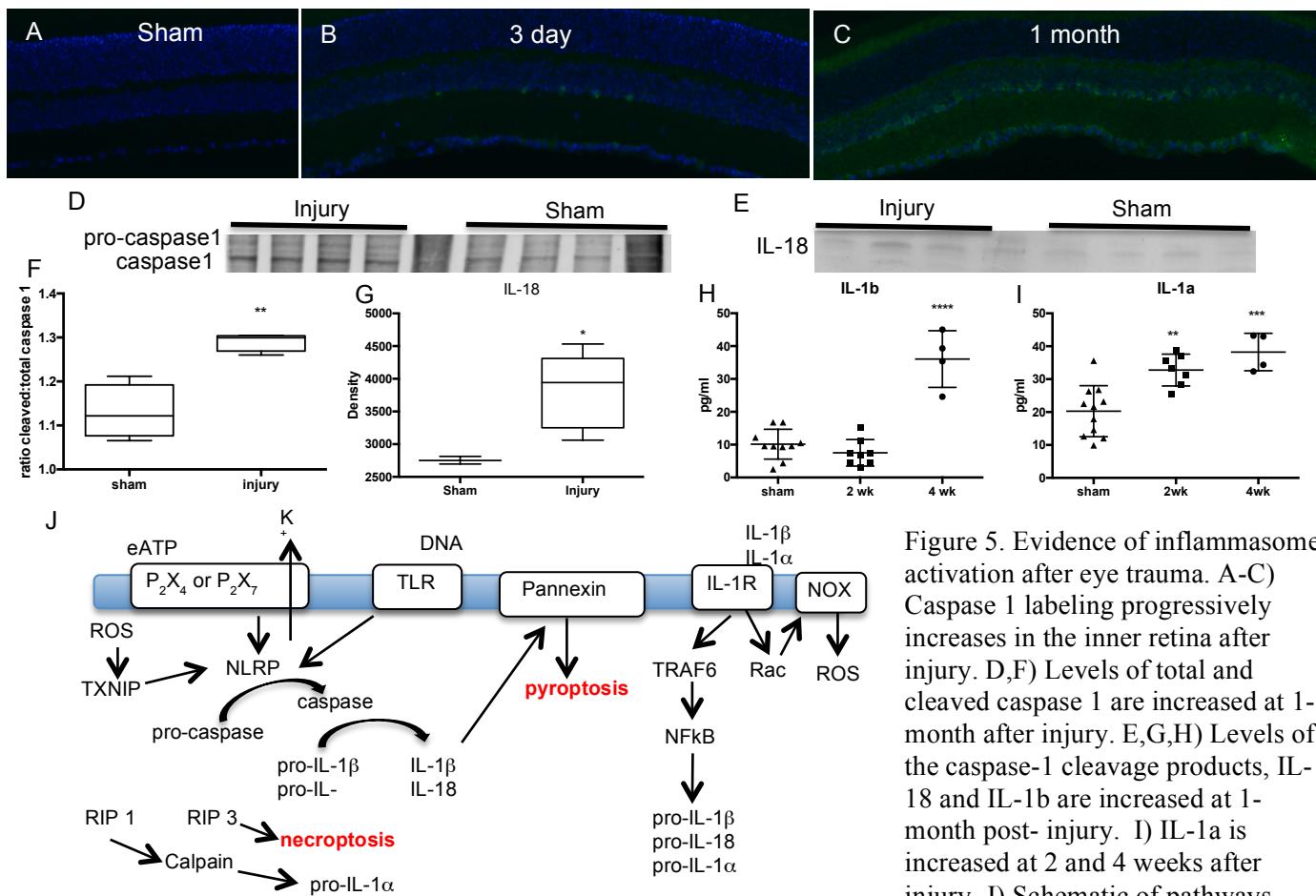


Figure 5. Evidence of inflammasome activation after eye trauma. A-C) Caspase 1 labeling progressively increases in the inner retina after injury. D,F) Levels of total and cleaved caspase 1 are increased at 1-month after injury. E,G,H) Levels of the caspase-1 cleavage products, IL-18 and IL-1b are increased at 1-month post- injury. I) IL-1a is increased at 2 and 4 weeks after injury. J) Schematic of pathways.

IL-1alpha and IL-1beta act through the IL-1R. We are producing a recombinant adeno-associated virus that we will use to provide sustained delivery of the IL-1R antagonist in the retina.

4. Impact: Any protective effect of a high Vit. C and Vit. E diet or a ketogenic diet is mild at best based on these preliminary data, suggesting that this approach would not be worthwhile to implement into the military MRE. However, the studies have illuminated a potential molecular pathway that may be responsible for the retinal ganglion cell degeneration: activation of sterile inflammation including an increase in the pro-inflammatory cytokine, IL-1 alpha. We are hopeful that blocking this pathway will be have greater protective efficacy than the antioxidants attempted in this study.

5. Changes/Problems: We were unable to perform animal studies for three months because the animal facility decided to treat all mice for fur mites. The treatment included the oxidant, hydrogen peroxide, which would clearly affect our studies. It then took time to ramp up our animal colony so that we could re-initiate the studies. In addition, two staff members left the laboratory unexpectedly and it has taken a few months to replace them. The laboratory is once again fully staffed.

6. Products: N/A

7. Participants and Other Collaborating Organizations: N/A

8. Special Reporting Requirements: N/A

9. Appendices: N/A